SUBSTRATE AND ANTIBIOTIC BINDING SITES AT THE PEPTIDYL TRANSFERASE CENTRE OF *E.COLI* RIBOSOMES

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1. Introduction

A number of antibiotic inhibitors of protein synthesis interfere with the function of the peptidyl transferase centre on the larger ribosomal subunit [1: review]. One of the mechanisms by which such inhibitors might act is competition for substrate-binding at the A-site or the P-site on the peptidyl transferase centre. In order to investigate this possibility, and to analyse further the mechanism of the peptidyl transfer reaction, we have developed assays for substrate-binding. There is evidence that only the terminal moeities of tRNA interact with the peptidyl transferase centre (fig. 1), and that other parts of the tRNA molecules perform other functions. In order to be specific, an assay for substrate-interaction at the centre should therefore employ substrates containing only the terminal portion of tRNA. The present paper reports an assay for substrate-interaction at the P-site, which is based on the measurement of binding of CACCA-Leu-Ac to 50 S subunits in the presence of ethanol. The interaction is blocked by certain antibiotic inhibitors of peptidyl transferase but not others. In conjunction with studies on binding at the A-site [6–8], results suggest that a number of antibiotics act at both the A-site and the P-site of peptidyl transferase while others act only at the A-site. A preliminary report of this work has been presented elsewhere [7].

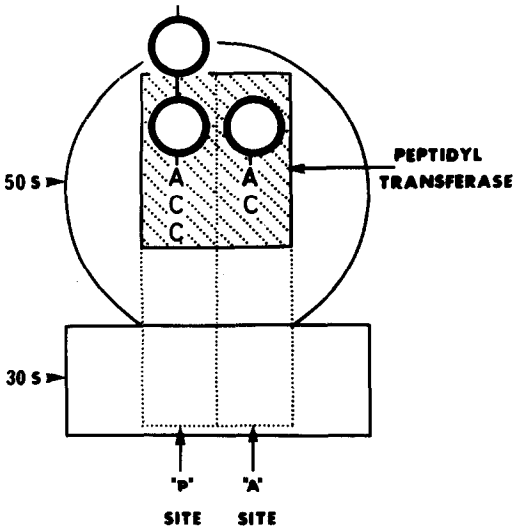

Fig. 1. Diagrammatic representation of information available on substrate interactions at the peptidyl transferase centre of the 50 S subunit [2]. Studies on the fragment reaction [3] suggest that interaction at the *P-site* of peptidyl transferase involves the terminal CpCpA of tRNA (but not other nucleotide residues in the tRNA), and is favoured by acetylation of the α-amino group. Studies on activity of substrates as peptidyl acceptors [4,5] indicate that interaction at the *A-site* of peptidyl transferase involves the terminal CpA of tRNA.

2. Materials and methods

2.1. *Materials*

Ribosomal subunits were prepared from log phase *E. coli* MRE 600 by the method of Staehelin [9]. CACCA-(^{3}H)Leu-Ac was prepared as in [3]. Sources of antibiotics were as previously indicated [10].

2.2. *Assay of CACCA-Leu-Ac binding*

The standard incubation mixture contained

Table 1
Binding of CACCA-Leu-Ac to ribosomal subunits and effect of streptogramin A.

Type of ribosomal subunit	Percentage of added CACCA-Leu-Ac bound to ribosomes		
	No addition	Plus 0.1 mM Streptogramin A	Difference
50 S	50	15	35
30 S	18	19	−1

Conditions and method of assay were as in text.

CACCA-(^{3}H)Leu-Ac (*c* 2 nM; specific activity *c* 20 Ci/mmole), 3 mg/ml 50 S subunits, 13 mM Mg acetate, 0.27 M KCl, 40 mM tris-HCl (pH 7.4), and 50% (v/v) ethanol. 150 μl aliquots were incubated in conical tubes at 0°C for 15–30 min and then centrifuged at 3000 *g* for 20 min at about 4°C (high speed centrifugation was used on samples lacking alcohol, and for time course experiments). 100 μl of the supernatant were mixed with scintillation fluid (Brays' solution [11] with 4% CAB-O-SIL) and radioactivity determined. Parallel incubations without ribosomes were included for estimation of total radioactivity under identical quenching conditions. The amount of bound substrate was calculated by difference.

3. Results

3.1. *CACCA-Leu-Ac binding to 50 S subunits*

There is no detectable interaction between CACCA-Leu-Ac and 50 S subunits under normal ionic conditions in absence of alcohol. This is not surprising in view of the requirement for alcohol in the ribosome-catalysed reaction of CACCA-Leu-Ac with puromycin (the "fragment reaction") [2,12]. Under the conditions of the fragment reaction (33% ethanol and 1 mg/ml ribosomes) there is a weak, but significant binding of CACCA-Leu-Ac to 50 S subunits. Binding can be enhanced by raising the concentrations of alcohol and ribosomes. The system used in most of our experiments contained 3–6 mg/ml of 50 S subunits and 50% ethanol. At higher concentrations of alcohol and ribosomes more binding takes place, but there is an unfavourable increase in the ratio of non-specific to specific binding.

In the experiment in table 1, 50% of the added CACCA-Leu-Ac bound to 50 S subunits. Addition of 0.1 mM streptogramin A lowered the binding to 15%. (In later experiments (e.g. table 2) streptogramin A-insensitive binding was less). Replacement of 50 S by 30 S subunits led to reduced binding and loss of sen-

Table 2
Binding of CACCA-Leu-Ac to 50 S subunits: effects of antibiotics.

Antibiotic	Concn. (mM)	Binding of CACCA-Leu-Ac (% of control)
Streptogramin A	0.1	7.6
Spiramycin III	0.1	12
Carbomycin	0.1	12
Lincomycin	1	36
Chloramphenicol	1	126
Celesticetin	1	123
Oleandomycin	1	125
Erythromycin	1	156
Viridogrisein	1	160
Sparsomycin	0.1	275
Gougerotin	1	230
Amicetin	1	193
Tetracycline	0.1	150

Edeine (2 μM), polydextran sulphate (5 μM), and anisomycin (0.1 mM) were without significant effect. Assay was as in text. Results are a complication of data from several assays. "Blanks" (streptogramin A-insensitive binding) were lower than in table 1, owing to the use of improved preparations of CACCA-Leu-Ac and 50 S subunits.

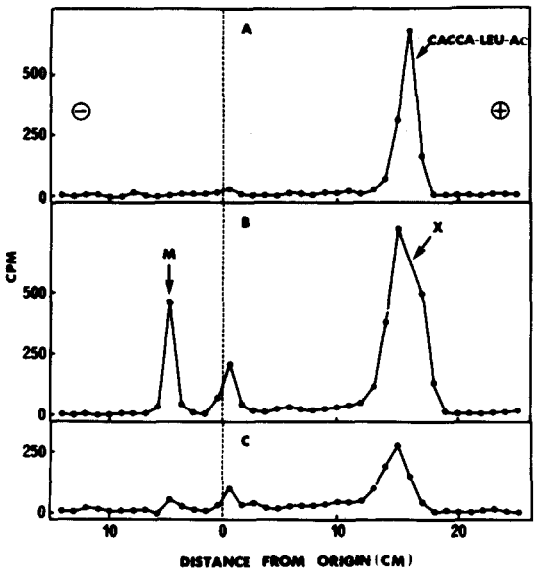

Fig. 2. Ionogram (pH 6.5) of (a) CACCA-(^{3}H) Leu-Ac marker, (b) eluate from complex of CACCA-(^{3}H) Leu-Ac and 50 S subunits, and (c) as "b" but from complex formed in presence of 0.1 mM streptogramin A. The incubation conditions for complex formation were as in text (2.2) but with 7 nM CACCA-Leu-Ac, 3 mg/ml 50 S subunits and a volume of 0.4 ml. After incubation, the sample was centrifuged and the sediment dissolved in 0.6 ml of 0.5 mM Mg acetate. The ribosome solution was incubated for 5 min at 25°C (to ensure disruption of the complex), and centrifuged at 50,000 rpm for 3 hr. The supernatant was lyophilized, re-dissolved in water and ionphoresed on Whatman 52 paper at pH 6.5 for 1 hr at 55 V/cm. One cm strips were cut out, immersed in scintillation fluid, and radioactivity estimated. Approximately 57% of the added CACCA-Leu-Ac was bound to ribosomes in the complete system and 20% in the incubation with streptogramin A. All of the bound radioactivity was recovered in the ribosome eluate, and all of the radioactivity applied to the ionogram was recovered in the area shown in the figure.

sitivity to streptogramin A. We conclude that the majority of the CACCA-Leu-Ac binding to 50 S subunits took place at a streptogramin A-sensitive site. The remainder of the binding to the 50 S subunits, as well as the binding to 30 S subunits, was probably due to non-specific interaction. In view of the potent inhibitory action of streptogramin A on the fragment reaction [13], it is reasonable to suppose that the streptogramin A-sensitive binding represents interaction of CACCA-Leu-Ac at a site on the peptityl transferase centre.

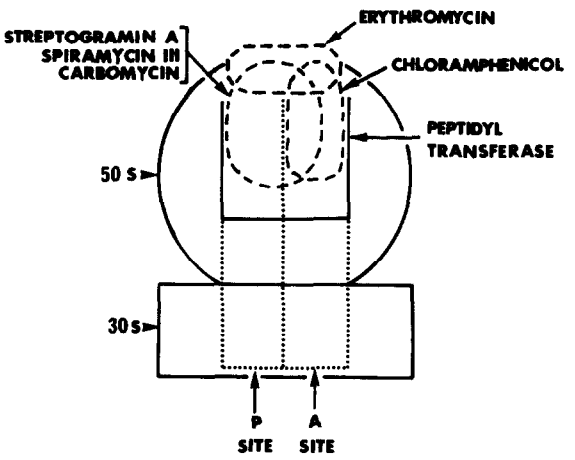

Fig. 3. Proposed sites of action of certain peptidyl transferase inhibitors. The antibiotics in this diagram are specific to 70 S ribosomes and compete with one another for binding to the 50 S subunit of *E. coli* ribosomes [1: review; 8]. All except erythromycin inhibit the fragment reaction. (Erythromycin actually reverses the inhibitory action of chloramphenicol on the fragment reaction [7], and of other macrolides and lincomycin on protein synthesis [15]). Present results suggest that the indicated antibiotics prevent substrate-interaction at the P-site. The same antibiotics, plus chloramphenicol, also exert an inhibitory influence on substrate-interaction at the A-site [6–8]. These various effects may be due to overlapping binding sites, as implied in the diagram, but other explanations are also possible (see text).

3.2. *Nature of the complex*

Interaction of CACCA-Leu-Ac with 50 S subunits takes place rapidly: equilibrium is reached in less than 10 min at 0°C. The complex can be completely dissociated by resuspension of the ribosomes in buffer without alcohol. To identify the eluted material, the complex was formed in the standard system, with and without streptogramin A, and then isolated, dissociated, and the eluate characterized by paper ionphoresis. Fig. 2 shows that at pH 6.5 approximately 70% of the radioactivity migrated as a single band, "X", at the same rate as a CACCA-Leu-Ac marker. About 18% of the radioactivity migrated as a minor band, "M", and 10%, remained stuck at the origin. (The component, "M", might be a degradation product, such as Ac-Leu-adenosine, but this has not been further investigated). The eluate from the complex formed in presence of streptogramin A contained the same components in very reduced amounts. We con-

conclude that the complex consists primarily of CACCA-Leu-Ac bound to the 50 S subunit by non-covalent bonds, and that the majority of the bound substrate is at a streptogramin A-sensitive site, presumably on the peptidyl transferase centre.

3.3. *Effects of antibiotics*

Table 2 shows the effects of various peptidyl transferase inhibitors [13] on the binding of CACCA-Leu-Ac to 50 S subunits. Spiramycin III and carbomycin were about as effective as streptogramin A in preventing complex formation. Lincomycin was also inhibitory. In contrast, complex formation was weakly stimulated by chloramphenicol, celesticetin, and oleandomycin, more strongly stimulated by erythromycin, viridogrisein and amicetin, and strongly stimulated by sparsomycin and gougerotin. Complex formation was unaffected by edeine and polydextran sulphate, inhibitors of 30 S subunit function [1], or by anisomycin, a specific inhibitor of 80 S ribosomes [1,14].

4. Discussion

There is evidence that chloramphenicol interferes with substrate-interaction at the A-site on the peptidyl transferase centre [6–8]. The failure of chloramphenicol to inhibit complex formation in the present system therefore suggests that CACCA-Leu-Ac did not bind at the A-site. Since the only other defined substrate-binding site on the centre is the P-site, and since CACCA-Leu-Ac acts as a good peptidyl donor under the conditions employed (unpublished data), it is probable that the binding of CACCA-Leu-Ac to 50 S subunits represents interaction at the P-site on the peptidyl transferase centre. As a corollary we may suppose that the antibiotics which inhibit such binding act at the P-site.

Fig. 3 shows a diagrammatic interpretation of the present results in conjunction with other current knowledge concerning the sites of action of peptidyl transferase inhibitors in *E. coli*. The model is only intended to be suggestive. Streptogramin A, spiramycin III, carbomycin, and possibly lincomycin block the P-site and also exert an inhibitory action on substrate-binding at the A-site. Chloramphenicol has an inhibitory action on substrate-binding at the A-site but not the P-site. Erythromycin acts at site which overlaps the binding sites of the above anitibiotics but not of the substrates. The actions of sparsomycin, amicetin and gougerotin are discussed elsewhere [7, 16, 6]. The action of any one of these antibiotics on the binding of other antibiotics or of substrates does not necessarily occur through direct competition at overlapping binding sites as implied in fig. 3, but might be the result of binding at sites which are spatially-separated but allosterically-linked. We are inclined to think that streptogramin A, spiramycin III and carbomycin compete directly with substrate for binding at the P-site, but recent evidence [8] suggests that chloramphenicol and lincomycin do not act in this manner on substrate-binding at the A-site. Work is in progress to define more clearly the nature of the substrate and antibiotic binding sites on the peptidyl transferase centre.

Acknowledgements

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